AN ABSTRACT OF THE THESIS OF

David B. Buchwalter for the degree of Master of Science in Toxicology presented on September 28, 1993.

Title: Modulation of Cupric Ion Activity by pH and Fulvic Acid as Determinants of Toxicity in Xenopus laevis Embryos and Larvae.

Redacted for Privacy

Abstract approved: _____
Lawrence R. Curtis

An ion specific electrode measured fulvic acid and pH modulated cupric ion activity in a series of modified frog embryo teratogenesis assay-xenopus (FETAX) toxicity assays. Hydrogen ion concentration was the primary determinant of cupric ion activity, while fulvic acid played a smaller but significant role. Fulvic acid (FA) was a weak copper complexing agent at pH 5.50. At pH 5.50 there was slight reduction of ionic activity and a subsequent attenuation of copper toxicity with 5.0 mg/l FA. At pH 7.50, FA also had a mild attenuating effect on copper toxicity. At pH 6.50, copper was strongly complexed by FA at total copper (TCU) concentrations below its pH dependent solubility limit. At total copper concentrations above the solubility limit FA enhanced toxicity. There was more cupric ion activity measured in the presence of 0.5 and 5.0 mg/l FA than without

it at total copper concentrations above the solubility limit. The proposed mechanism for this behavior was FA action as a nucleation inhibitor under these chemical conditions, which resulted in a stable supersaturation of copper, and a more toxic aqueous matrix.

Key words: copper; toxicity; FETAX; fulvic acid; pH; ion specific electrode; activity; MINTEQ

Modulation of Cupric Ion Activity by pH and Fulvic Acid as Determinants of Toxicity in Xenopus laevis Embryos and Larvae.

by

David B. Buchwalter

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Completed September 28, 1993

Commencement June 1994

APPROVED:

Redacted for Privacy

Professor of Toxicology, in charge of major

Redacted for Privacy

Chair of Toxicology Program

Redacted for Privacy

Dean of Graduate School

Date thesis is presented September 28, 1993

Typed by David Buchwalter

ACKNOWLEDGEMENT

The author would like to thank Larry Curtis and Greg Linder for their input and help in editing this document, Sueann Ott for metals analysis, Henry Lee for statistical help, and David Wilborn for his many helpful suggestions.

Special thanks to Dr. John Westall, for his invaluable assistance and guidance.

Thanks also to Mike Bollman and David Schmedding for their support of this project.

TABLE OF CONTENTS

INTRODUCTION 1

MATERIALS AND METHODS 4
Dilution water 4
Preparation of Metal Stock Solutions 4-5
Preparation of Fulvic Acid Stock Solutions 5
Preparations of Treatment Groups 5-6
Frog Culture and Preparation of the Eggs 6-7
Exposures 7-8
Biological Responses 8
Total Metal Analysis 8-9
Cupric Ion Activity 9-10

RESULTS 12
Probe Response 12-16
Biological Responses 16-20
Malformations 20-25

DISCUSSION 26

CONCLUSION 31

BIBLIOGRAPHY 32

APPENDICES 35

Appendix 1. 35
Dissolved Organic Material, Humic and Fulvic Acids

Appendix 2 38
Bioassessment

Appendix 3. 39
Dilution Water

Appendix 4 40
pH Drift

Appendix 5 41
Interactions

LIST OF FIGURES

Figure		Page
1.	Cupric ion activity calibration curve	11
2.	Cupric ion specific electrode millivolt measurements	13
3.	Comparison of ISE replicates at pH 6.50	15
4.	Mortality	17
5.	Growth inhibition	18
6.	Malformation	21
7.	Three proposed average structural models of fulvic acid	37

LIST OF TABLES

Table		Page
1.	Pooled data for two replicates at pH 5.50	23
2.	Pooled data for two replicates at pH 6.50	24
3.	Results of an additional replication of the 6.50 experiments	25
4.	Relative abundance of humic materials in surface waters	36
5.	pH drift	40
6.	Interactions	41

MODULATION OF CUPRIC ION ACTIVITY BY pH AND FULVIC ACID AS DETERMINANTS OF TOXICITY IN XENOPUS LAEVIS EMBRYOS AND LARVAE.

INTRODUCTION

Many water resources receive wastes from anthropogenic sources. Mining activities and various industrial processes produce heavy metal wastes such as copper that have created ecotoxicological problems. The application of copper to aquatic ecosystems for the control of blue-green algae is still commonly practiced today. Because copper is so widespread in both polluted and unpolluted waters, there is abundant literature on copper toxicity.

Although it is widely accepted that copper toxicity is dependent on the chemistry of a given system, there is a surprising lack of literature that examines the interaction of physicochemical parameters with copper toxicity. Water hardness, pH, and dissolved organic material exert profound influence on the toxicity of copper. For example, increasing water hardness reduces the toxicity of copper to aquatic vertebrates (USEPA, 1980). This presumably results from calcium ions competing with metal ions for binding sites on, for example, the gills of fish. The hydrogen ion

concentration in aquatic systems is the primary determinant of solubility, speciation and availability of many heavy metals (Stumm and Morgan, 1981). Copper solubility is extremely pH dependent, and higher solublility and toxicity are associated with low pHs. Dissolved organic materials (DOM) such as humic acids (HA) and fulvic acids (FA), are known to interact with heavy metals, and in many cases reduce metal availability (Winner 1984, 1985).

From a biological perspective, the relationships among pH, water hardness, dissolved organic material and metal toxicity have been investigated with fish, algae and daphnids. Much biological work has not adequately characterized the chemistry of the test system, resulting in incomplete or marginally useful data. On the other hand, purely chemical approaches are often conducted under conditions that do not approach those of natural waters, and may not extend to consequences of physicochemical interactions in ecosystems. This fragmentation of research into purely biological or chemical experimentation leaves gaps in our understanding of pollutant interactions in aquatic ecosystems.

There have been numerous chemical studies of the complexation of heavy metals by DOM including HA (Schnitzer and Kerndorff, 1981; Hering and Morel, 1988; Dzombak et al., 1986; McKnight et al., 1983; Tipping, 1993; Buffle et al., 1977). Few studies, however, have quantified the degree to

which complexation and changes in metal speciation by DOM affects the toxicity of these metals (Meador, 1991; Meador et al., 1993). Humic substances (HA and FA) attenuate metal toxicity in some studies (Shanmukhappa and Neelakantan 1990, Gundersen et al. personal communication 1991) while toxicity varied depending on the concentrations of metal and HA and other factors including hardness, alkalinity, and pH in others (Stackhouse 1989; Garvey et al. 1991; Stackhouse and Benson 1988; Winner 1986; Winner and Gauss 1986). Winner (1986) described enhanced cadmium toxicity with HA under certain chemical conditions, but did not propose a mechanism for this unusual response. This study was designed to investigate the toxicity of copper within defined chemical conditions. Specifically, changes in cupric ion activities resulting from physicochemical interactions of pH and fulvic acid were measured. These activities are correlated with biological responses of the South African Clawed Frog- Xenopus leavis.

MATERIALS AND METHODS

In this study, two concentrations of Suwannee River fulvic acid (obtained from the International Humic Substances Society) were used. Nominal concentrations of 0.50 mg/l FA and 5.00 mg/l FA, representing relatively low and high surface water levels, were used to simulate lake, stream and river water environments. Copper concentrations were chosen to target non-lethal endpoints, though some mortality did occur. Growth inhibition and terata were also examined. Measurements of total copper (TCU) and cupric ion activity were made for correlation with toxicity.

Dilution water. Reconstituted water was used for all experiments as that described by Birge et al. (1979) (Appendix 2). A 1 X 10^{-3} molar phosphate buffer was used to control the hydrogen ion concentration for all exposure regimes.

Preparation of metal stock solutions. Copper was purchased from Sigma Chemical Company (St. Louis, MO) as the chloride salt. Stock solutions of 10 mg/l were prepared with the dilution water described above. The dilution water was filtered through a Nuclepore® 0.45 um mixed ester cellulose

filter prior to the addition of metal. One ml of concentrated Baker® nitric acid was added per liter of dilution water to prevent the precipitation of copper hydroxides.

Preparation of fulvic acid stock solutions. Suwanee River FA reference material was purchased from the International Humic Substances Society (IHSS) (Denver, CO). Stock solutions of 20 mg/l were prepared with the filtered dilution water as described above. Elemental analysis of the FA was provided by the IHSS. The means of seven replicates were: 53.493% carbon, 4.290% hydrogen, 41.020% oxygen, 0.696% nitrogen, 0.560% sulfur, and 0.854% ash. These values are reported on an ash-free, moisture-free basis (Reddy et al., 1989 as cited in USGS open file report 87-557). Aiken et al. (1989 as cited in USGS open file report 87-557) used small-angle X-ray scattering, vapor-pressure osmometry, equilibrium ultracentrifugation, and fast atom bombardment mass spectroscopy and determined the average molecular weight of Suwanee River FA was about 800 daltons.

Preparations of treatment groups. For each treatment group, copper and FA stock solutions were pipetted into Nalgene® polycarbonate 280 ml containers, and brought to 270 ml with filtered dilution water. Prior to pH adjustment, each solution was acidified with concentrated HCl to prevent

precipitation, and stirred well to obtain 10 ml samples for matal analysis. All solutions were then brought to the desired final pH with NaOH.

Frog culture and preparation of the eggs. Eggs were obtained from the Xenopus colony at EPA's Environmental Research Laboratory - Corvallis, Oregon. Adult frogs were kept in 36.5 X 11 X 16 inch glass aquaria in an environmentally controlled chamber. A temperature of 24.0 +/-1.0° degrees celsius was maintained with a 16:8 hr light:dark photoperiod. Frogs were fed a diet of beef liver twice weekly, and their water was changed after feeding. Well water from EPA's Willamette Research Station in Corvallis was used in frog holding and breeding tanks.

Adult frogs were induced to breed via an injection of human chorionic gonadotropin (Sigma, St. Louis MO) in the dorsal lymph sack (ASTM, 1993). The eggs were then harvested, and the jelly coats were removed with a 2% l-cysteine solution in distilled water. This solution was adjusted up to pH 8.10 with 10% NaOH. This procedure was performed as specified in ASTM (E 1439-91), with the exception that dilution water was used rather than FETAX solution. The de-jellied eggs were then sorted under a dissection microscope; only normally cleaving eggs were transferred to a 100 mm diameter petri dish with dilution

water. Stage 8 1/2 blastulae were used at the start of each experiment (Nieuwkoop and Faber, 1975).

Exposures. A series of five copper concentrations, plus controls were prepared with the dilution water mentioned above. Exposure chambers were 60 x 15 mm plastic petri dishes. For each copper concentration, nine exposure chambers were used (Figure 1). Fifteen eggs were placed in each chamber. For each concentration of copper, three replicates were run FA, three with 0.50 mg/l FA, and three with 5.00 mg/l FA. This array was run at nominal pH of 5.50, 6.50, and 7.50. The pH was measured with an Omega PHB-70X water analyzer (Stamford, CT), and adjustments in exposure media pH were made daily with HCl and NaOH as needed. Previous investigation determined that between the pH 5.50 and 7.50, Xenopus embryos and early larval stages developed without observed hydrogen ion toxicity. Exposures occurred at 24° +/- 1.0° C in an environmental chamber with a 16:8 hr light:dark photoperiod. Because of limitations in the number of eggs available, each FA-metal treatment was conducted at one pH block at any time. Each experiment was replicated for each pH.

All exposure media were equilibrated for 5-8 days prior to the first exposure day. During this time, the pHs were adjusted daily with NaOH or HCl as needed. When pH drift in the treatment groups was minimal, exposures were initiated.

The pHs of every exposure group were taken daily prior to the transfer of media to individual petri dishes. Mortalities were recorded and removed daily. After living eggs and larvae were transferred to new media, the old media was pooled from the three dishes, and the pHs were recorded. The eggs were transferred to a new dish containing 10 ml of exposure media every 24 hr. Exposures lasted 96 hr.

Biological responses. After 96 hr of exposure, surviving larvae were examined with a dissection microscope for developmental abnormalities. For control groups and the lowest exposure groups it was necessary to anesthetize the larvae with MS-222. Terata were recorded for each dish. Growth inhibition was examined by measuring the individual larvae with the Sigma Scan scientific measurement system (Jandel Scientific, Corte Madera, CA). A photographic darkroom enlarger was used to project the image of the larvae onto a digitizing pad, and the individual images were traced with a mouse to read lengths into a data file.

Total metal analysis. Copper concentrations of test solutions were determined with a A Bausch and Lomb/Applied Research Laboratory model 3580-vacuum inductively coupled plasma-atomic emission spectrometer (ICP-AES). Dilution

water used for control groups was also analyzed by ICP-AES to measure background metals.

Cupric ion activity. An Orion (Boston, MA) cupric ion specific electrode (model number 94-29) was used with an Orion (model 90-02) double junction reference electrode, with 10% KNO_3 as the outer chamber filling solution to obtain millivolt measurements to +/-0.1 mV. The slope of the probe response was checked prior to experimental measurements to insure the response was within the range 25-30 mV/decade as described by Orion. Millivolt measurerments were taken in unstirred conditions, and values were not recorded until readings were stable for at least three minutes. This usually took 20-35 minutes to reach stability. After all solutions were measured, pH measurements were taken again to insure that all pHs were within +/- .04 pH units.

The MINTEQA2/PRODEFA2 (Allison et al. 1990) geochemical assessment model simulated inorganic equilibria for copper in each zero FA treatment. These simulations established a speciation profile for the five metal concentrations at each pH. Model generated estimates of cupric ion activitiy were available in conjunction with cupric ion specific electrode data. The simulations provided (1) a method for determination of cupric ion activity in organic-free

solutions, and (2) a tool to complement the ion specific electrode in determining cupric ion activities in the fulvic acid treatments.

Cupric ion activity was estimated from MINTEQ model simulations of zero fulvic acid treatment groups at pH=5.50 and 6.50. The corresponding millivolt measurements taken from those same groups was used to generate a calibration curve from which all other millivolt measurements could be assigned a corresponding activity (Figure 1).

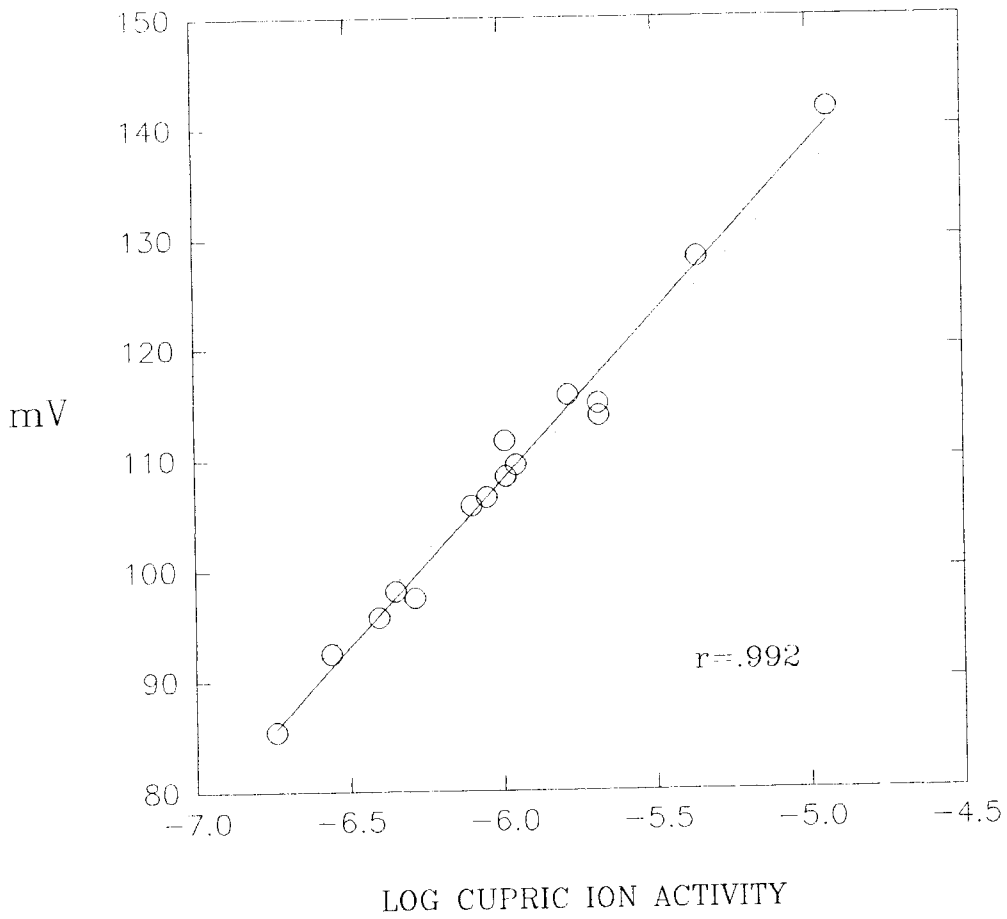


Figure 1. Cupric ion activity calibration curve. Activity estimates were obtained from MINTEQ modelling output. Millivolt data were obtained from an Orion cupric ion specific electrode.

$$mV = 289.675 + 30.266(\text{Log activity})$$
$$(6.169) \quad (1.027)$$

RESULTS

Probe response. Regression models for millivolt measurements at pH=5.50 versus total copper were simultaneously fit for the zero, 0.5, and 5.0 mg/l FA treatment groups using indicator variables (Neter and Wasserman, 1983). The only significant difference between the groups was that the equation for the 5.0 mg/l FA is shifted 5.3 millivolts lower that the 0.0 and 0.5 mg/l FA (p=.036)(Figure 2a). The regression model was quadratic estimated as:

$$\text{0.0 and 0.5 mg/l FA:}\quad mV = \underset{(2.0)}{92.0} + \underset{(13.3)}{122.1}\,TCU - \underset{(11.4)}{69.7}\,TCU^2.$$

$$\text{5.0 mg/l FA:}\quad mV = \underset{(2.4)}{86.7} + \underset{(13.3)}{122.1}\,TCU - \underset{(11.4)}{69.7}\,TCU^2.$$

The 5.3 millivolt shift corresponds with a decrease in Log cupric ion activity of 0.1726.

At pH 6.50 ISE measurements indicated strong complexation at TCU concentrations below the solubility limit of copper (Figure 2b). Voltages at both 0.5 and 5.0 mg/l were significantly lower than for the 0.0 mg/l FA groups up to TCU=0.435 +/- 0.002 mg/l. At that concentration, where MINTEQ modelling output suggested the system was essentially saturated with copper, and at 1.195 +/- 0.005 mg/l, the trend of complexation and lower

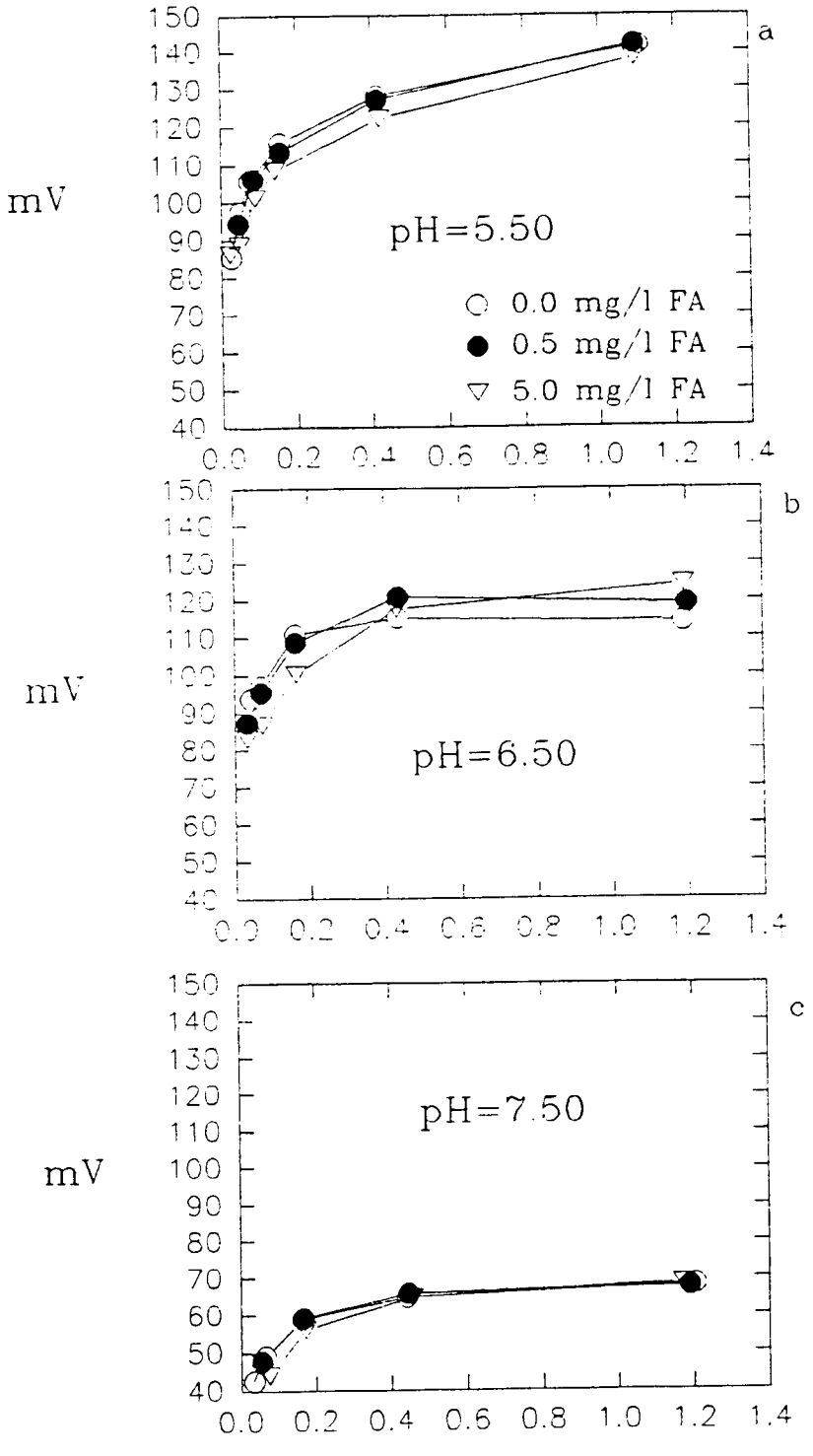


Figure 2. Cupric ion specific electrode millivolt measurements vs.total copper at pH a)5.50, b)6.50 and c)7.50.

activities reversed. Higher millivolt readings were recorded for the higher FA groups. This was opposite what was observed throughout the pH 5.50 experiments, and readings taken at below saturation concentrations at pH 6.50. ISE readings were taken again the following day, with good replication of voltage values. As these results were unexpected, the experiments were repeated, and the same patterns were observed (Figure 3).

Regression models for millivolt measurements at pH=7.50 (Figure 2c) versus TCU for the zero, 0.5, and 5.0 mg/l FA were simultaneously fit using indicator variables (Neter and Wasserman, 1983). There were no significant differences between the three groups. The regression model was a quadratic curve estimated as:

$$\begin{array}{lccc} mV = & 43.43 & + 83.48\ TCU & - 50.78\ TCU^2. \\ & (3.70) & (22.22) & (17.48) \end{array}$$

Because the measured voltages were so low at this pH, and response becomes nonlinear towards the detection limits of the probe, there was no attempt to use the linear calibration curve to convert these measurements to activities. There were too few data available to produce a curvalinear model to fit the probe response. Biological responses were reported in terms of total copper.

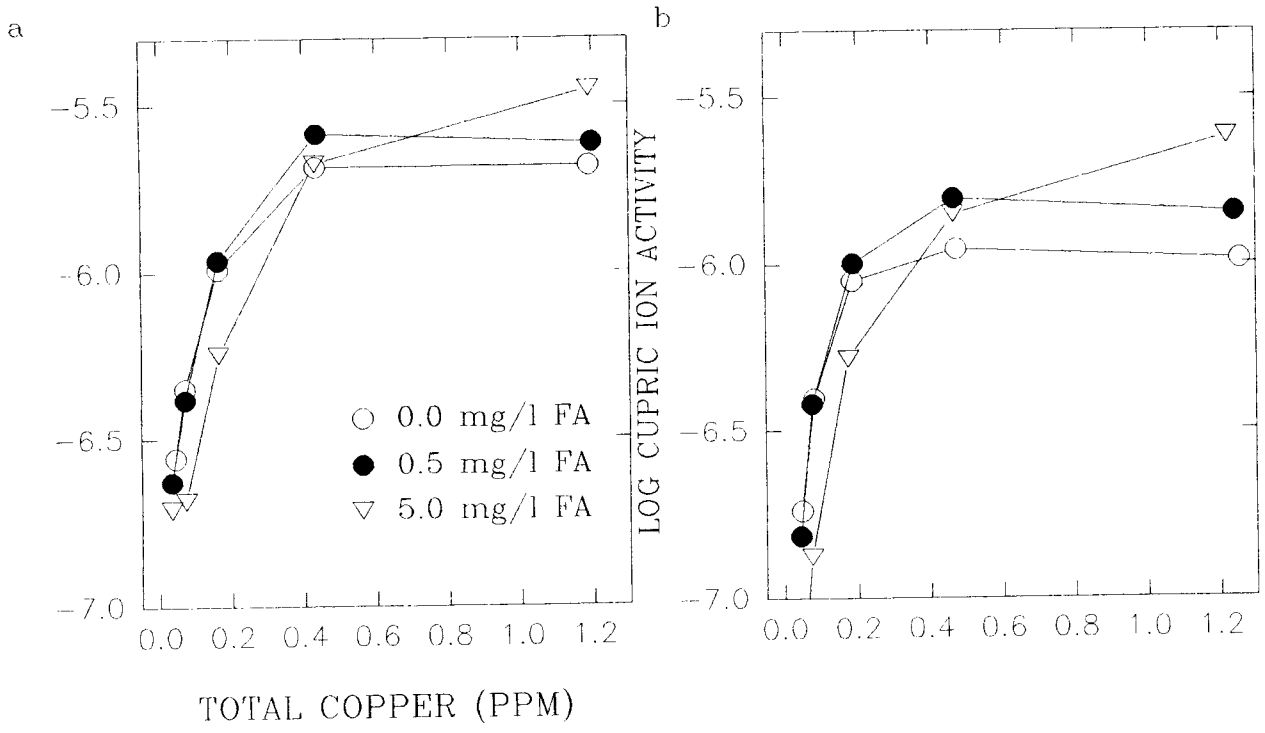


Figure 3. Comparison of ISE replicates at pH 6.50. Fulvic acid modulated cupric ion activity at pH 6.50. The differences in the magnitude of ionic activity are most likely due to the kinetics of the system.

Biological responses.

Mortality was very highly correlated with cupric ion activity over all pH treatments. There was no significant copper concentration-dependent mortality in any of the treatment groups at pH 7.50 (data not shown). A Weibull regression model was fit for activity vs. mortality data for two replicates at pH 5.50, and three replicates at pH 6.50:

$$\text{Mortality} = \underset{(24.60)}{139.85} * \exp\left[-\left(\log \text{activity}/\underset{(.08)}{-5.29}\right)^{\underset{(3.71)}{15.98}}\right]$$

R^2=.95 (Figure 4).

Length was also very highly correlated with ionic activity (Figure 5). There was no statistically significant growth inhibition at pH 7.50 (data not shown). Weibull regression models were individually fit for pH 5.50 and each 6.50 data set, and were as follows:

$$\text{pH 5.50}\quad \text{Length} = \underset{(.0039)}{0.9732} - \exp\left[-\left(\log \text{activity}/\underset{(.0246)}{-5.1793}\right)^{\underset{(1.5140)}{13.4358}}\right]$$

R^2=.98 (Figure 5a).

$$\text{pH 6.50a}\quad \text{Length} = \underset{(.0082)}{.99046} - \exp\left[-\left(\log \text{activity}/\underset{(.0269)}{-5.37987}\right)^{\underset{(2.7108)}{19.5537}}\right]$$

R^2=.92 (Figure 5b).

$$\text{pH 6.50b}\quad \text{Length} = \underset{(0.0087)}{1.044} - \exp\left[-\left(\log \text{activity}/\underset{(.1581)}{-5.1219}\right)^{\underset{(2.1762)}{8.1125}}\right]$$

R^2=.75 (Figure 5c).

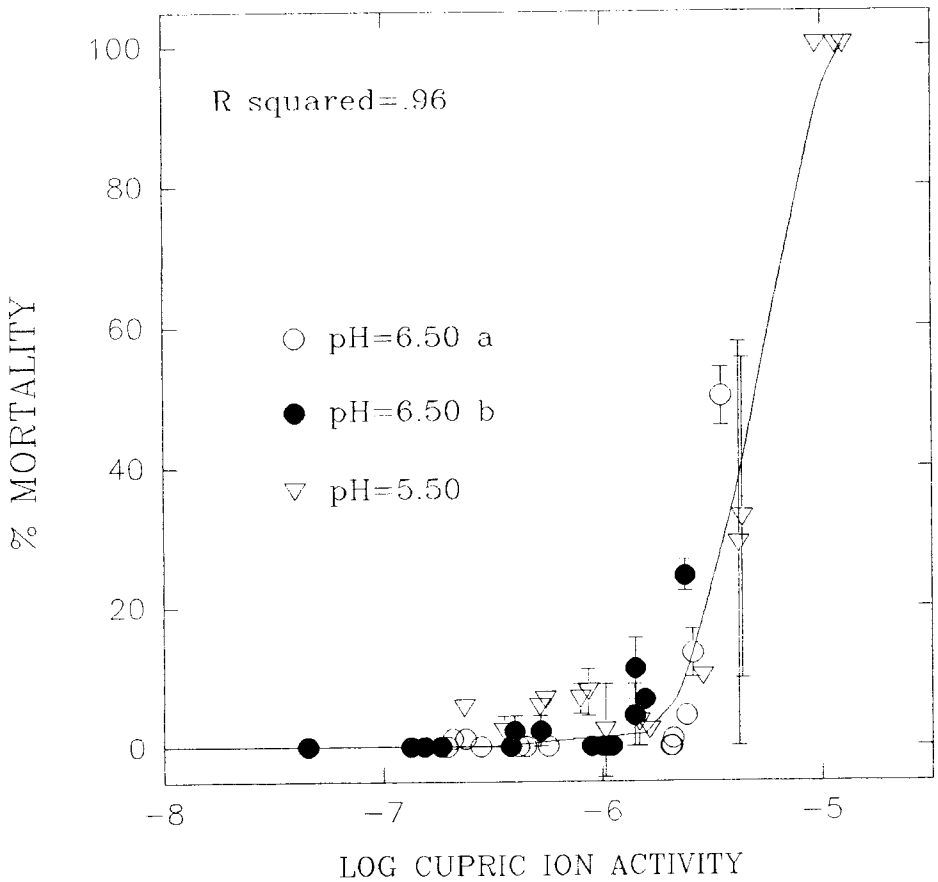


Figure 4. Mortality. A Weibull regression model was fit for pooled mortality data over the pH range of 5.50 and two replicates at 6.50. The model has the form:

$$\% \text{ mortality} = 139.85 * \exp[-(\text{Log activity}/-5.29)^{15.98}]$$

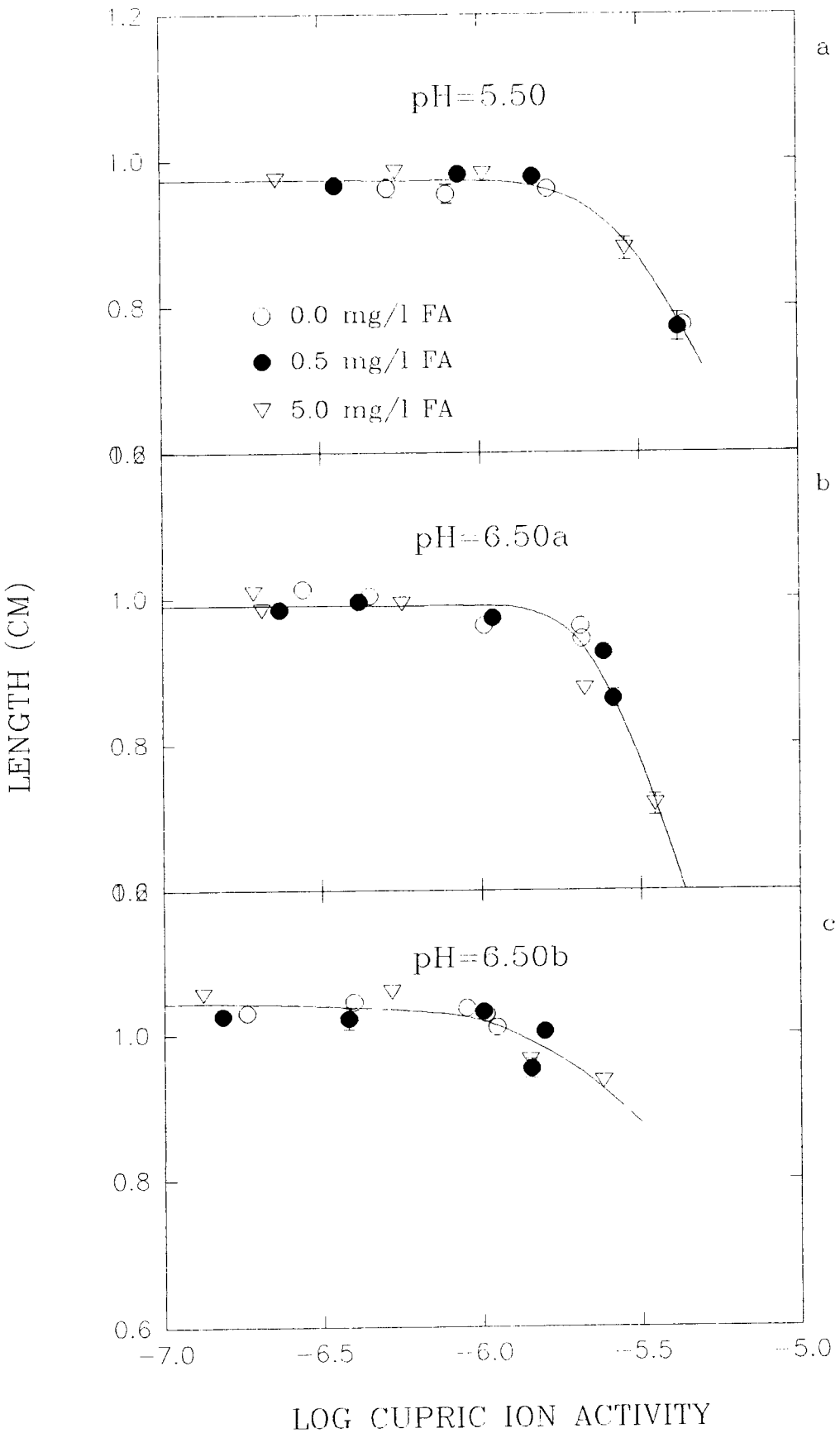
Figure 5. Growth inhibition. Comparison of growth inhibition data for pH a) 5.50, b) 6.50a and c) 6.50b. Error bars are the standard errors of the means. The fit curves are Weibull regression models of the form:

Length = $a-\exp[-(\text{Log activity}/b)^c$

a = asymptotic to no effect lengths

b = log activity associated with 37% response

c = a shape parameter


a
pH=5.50
○ 0.0 mg/l FA
● 0.5 mg/l FA
▽ 5.0 mg/l FA
b
pH=6.50a
c
pH=6.50b
LENGTH (CM)
LOG CUPRIC ION ACTIVITY
1.2
1.0
0.8
0.6
−7.0
−6.5
−6.0
−5.5
−5.0

A common model was fit for all data, with R^2=.81, but a test for the adequacy of a common model rejected the hypothesis that there was no difference between the full regression model, and the individual models described above.

Malformations. Slight facial malformations and incomplete gut coiling were observed for high TCU at pH 7.50, and were only statistically significant in few cases. At pH 5.50 and 6.50, malformation rates were highly correlated with cupric ion activity. Weibull regression models were fit for data over these pHs, (these were bound to a lower limit of 10% to be biologically realistic, as control malformation rates were approximately 10%) and were as follows:

pH 5.50

$$\% \text{ Malformed} = 10 + 90 * \exp[-(\log \text{activity}/-5.688)^{\frac{36.296}{(0.107)(28.64)}}$$

R^2=.86

pH 6.50a

$$\% \text{ Malformed} = 10 + 90 * \exp[-(\log \text{activity}/-5.678)^{\frac{91.56}{(0.018)(47.53)}}$$

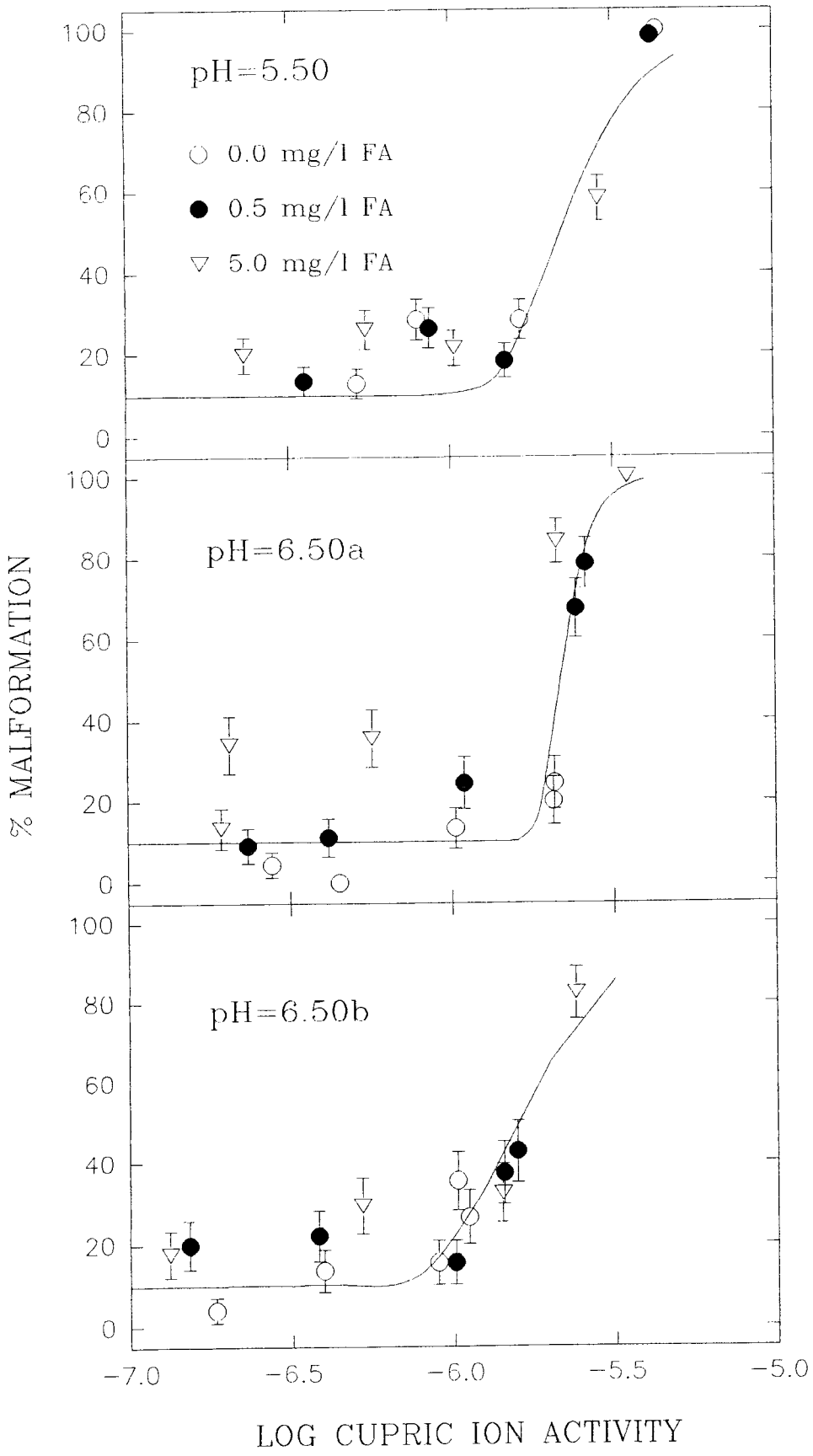
R^2=.74

pH 6.50 b

$$\% \text{ Malformed} = 10 + 90 * \exp[-(\log \text{activity}/-5.846)^{\frac{28.579}{(.025)\ (6.605)}}$$

R^2=.68

Figure 6. Malformation. Weibull type regression models were fit for malformation data for pH 5.50 and two replicates at pH 6.50. These models were bound so that the curves were asymptotic to 10% malformation as a lower limit, and 100% as an upper limit.


pH=5.50
○ 0.0 mg/l FA
● 0.5 mg/l FA
▽ 5.0 mg/l FA
pH=6.50a
pH=6.50b
% MALFORMATION
LOG CUPRIC ION ACTIVITY
100
80
60
40
20
0
−7.0
−6.5
−6.0
−5.5
−5.0

FULVICS (mg/l)	TOTAL COPPER (mg/l)	MALFORMATION %	MORTALITY %	LENGTH (cm)
0.0 mg/l	0.024	12.50 (3.52)	2.20 (<.01)	0.9550
	0.049	12.90 (3.64)	5.60 (1.00)	0.9630
	0.075	28.60 (4.93)	6.80 (2.11)	0.9550
	0.156	28.40 (4.81)	2.20 (<.01)	0.9610
	0.414	100.00 (0.00)	32.60 (28.87)	0.7740
	1.108	-	100.00 (0.00)	
0.5 mg/l	0.024	9.20 (3.09)	3.30 (3.33)	0.9890
	0.044	13.60 (3.66)	2.20 (2.22)	0.9660
	0.085	26.50 (4.84)	7.80 (3.33)	0.9810
	0.156	18.40 (4.15)	3.30 (3.33)	0.9780
	0.414	98.40 (1.55)	28.90 (28.88)	0.7710
	1.095	-	100.00 (0.00)	
5.0 mg/l	0.022	17.20 (4.05)	3.30 (1.11)	0.9750
	0.047	20.00 (4.34)	5.60 (1.11)	0.9740
	0.090	26.20 (4.79)	6.70 (6.66)	0.9840
	0.143	21.60 (4.39)	2.20 (<.01)	0.9810
	.422	58.20 (5.55)	9.90 (1.22)	0.8780
	1.096	-	100.00 (0.00)	-

Table 1. Pooled data for two replicates at pH 5.50. The standard error of the means are in parenthesis.

FULVICS (mg/l)	TOTAL COPPER (mg/l)	MALFORMATION (%)	MORTALITY (%)	LENGTH (cm)
0.0 mg/l	<0.012	2.30 (2.24)	1.10 (1.11)	.9910
	0.044	4.40 (3.07)	0.00	1.0130
	0.071	0.00	0.00	1.0030
	0.162	13.30 (5.06)	0.00	.9630
	0.434	20.00 (5.96)	0.00	.9610
	1.190	24.40 (6.40)	0.00	.9430
0.5 mg/l	<0.013	4.40 (3.07)	0.00	.9960
	0.033	9.10 (4.33)	1.10 (1.11)	.9840
	0.071	11.10 (4.68)	0.00	.9950
	0.162	24.40 (6.40)	0.00	.9730
	0.437	78.60 (6.33)	13.30 (3.44)	.8620
	1.199	67.40 (7.15)	4.40 (1.40)	.9250
5.0 mg/l	<0.014	6.70 (3.71)	0.00	1.0050
	0.033	13.30 (5.06)	0.00	1.0080
	0.073	34.10 (7.14)	1.10 (1.11)	.9840
	0.164	35.60 (7.13)	0.00	.9930
	0.433	84.10 (5.51)	1.10 (1.11)	.8740
	1.193	100.00	50.00 (4.13)	.7160

Table 2. Pooled data for two replicates at pH=6.50. Malformation data is for one replicate only. The standard error of the means are in parenthesis.

* Due to incomplete fixation of larvae, malformation data was only obtained for one replicate for this set of experiments.

FULVIC ACID (mg/l)	TOTAL COPPER (mg/l)	MALFORMATION (%)	MORTALITY (%)	LENGTH (cm)
0.0 mg/l	0.019	9.10 (4.33)	2.20 (2.22)	1.00900
	0.048	4.44 (3.07)	0.00	1.02997
	0.080	13.60 (5.17)	2.20 (2.22)	1.04550
	0.187	15.60 (5.40)	0.00	1.03651
	0.473	26.70 (6.59)	0.00	1.00991
	1.255	35.60 (7.13)	0.00	1.02854
0.5 mg/l	0.021	13.60 (5.17)	2.20 (2.22)	1.01307
	0.043	20.00 (5.96)	0.00	1.02558
	0.076	22.20 (6.19)	0.00	1.02177
	0.188	15.60 (5.40)	0.00	1.03127
	0.466	42.80 (7.63)	6.70 (0.00)	1.00394
	1.241	37.50 (7.65)	11.10 (4.44)	.95274
5.0 mg/l	<.015	18.20 (5.81)	2.20 (2.22)	1.06101
	0.038	8.90 (4.24)	0.00	1.05641
	0.074	17.80 (5.69)	0.00	1.05562
	0.175	29.50 (6.87)	2.20 (2.22)	1.05892
	0.465	32.60 (7.15)	4.40 (4.44)	.96334
	1.221	82.40 (6.53)	24.40 (2.22)	.93379

Table 3. Results of an additional replication of the 6.50 experiments. Standard errors of the means are in parenthesis.

DISCUSSION

The hydrogen ion concentration played a pivotal role in the activity and toxicity of copper. Not only was pH the primary determinant of cupric ion activity in the zero FA treatments, but it also determined FA behavior. Fulvic acid played a biphasic role in modulating ionic activity and toxicity.

At pH 5.50, FA was a mild cupric ion complexing agent. There was no difference in cupric ion activities between the zero and the 0.5 mg/l FA treatment groups, but at 5.0 mg/l FA there was significantly lower activity and a corresponding decrease in toxicity. At high total copper however, there was sufficient cupric ion activity to result in 100% mortality, regardless of FA. Perhaps in this acidic environment, many of the complexation sites on the FA molecule (carboxylic acids and phenolic groups) are in a protonated state, resulting in a weakly complexing form.

Contrary to the attenuation of toxicity by FA observed at pH 5.50, enhanced free ionic copper and resulting enhanced toxicity were observed at pH=6.50. ISE measurements shows free cupric ion concentrations well above the solubility limit in the zero FA groups. Figure 6 (a and b) show a variety of chemical interacions. In the zero FA treatment groups, ionic activity measurements show the

system tending towards saturation. At higher total copper concentrations, there was little change in ionic activity as excess copper precipitated. In the FA treatment groups at low total copper, there was very strong complexation of cupric ions. The degree of complexation was positively correlated with FA concentration. There was not however, an attenuation of toxicity in these low TCU treatments. In fact there were elevated biological responses in these groups. The mechanism for this was not identified.

At higher total copper concentrations however, the trend of complexation and subsequent lower ISE voltage measurements for FA were reversed. Where the zero FA system became saturated, the 0.5 and 5.0 FA treatment groups actually exhibited higher millivolt measurements. Expressed toxicity dramaticly increased corresponding with the higher ISE measurements. Increased mortality, malformation and growth inhibition were observed for the FA treatment groups in comparison to the zero FA groups.

An explanation of this behavior necessitates a discussion of precipitation dynamics, and more specifically nucleation. Nucleation can be described as the birth of crystals from solutions (Walton, 1967). This process controls the size, number and structure of precipitated crystals. "Prior to nucleation there is continuous formation and dissolution of ionic or molecular clusters in equilibrium with all other clusters. If the concentration

of solute ions or molecules is high enough, the clusters become significantly large enough to become consolidated into small crystallites, whereupon the supposedly irreversible crystal growth ensues" (Walton, 1967).

Certain compounds greatly hinder the formation of crystals, and hence stabilize supersaturated solutions. Very small quantities of these materials inhibit nucleation. For example, 0.3 ppm of polyacrylic acid hinders the formation of calcium sulfate crystals. Patents have been issued for compounds having two adjacent carboxylic groups and spaced along the polymeric chain, which prevent calcium carbonate deposition (Walton, 1967). Adjacent carboxylic acid groups may also occur in FA. Certainly carboxylic acid groups exist in close proximity on some FAs. FA could be functioning as a nucleation inhibitor under the chemical conditions at pH=6.50 (Westall, personal communication). This would account for the enhanced activity and toxicity as a function of increasing FA concentrations.

Winner (1986) described enhanced cadmium toxicity with the presence of HA at different water hardnesses, but did not propose a mechanism. The binding to, adsorption, or blocking of crystal growth sites has been proposed as a mechanism for inhibiting nucleation and crystal growth (van Rosmalen, Weijnen and Meijer, 1883; Nancollas and Zawacki, 1984). Industrial applications of this phenomenon are found in the prevention of scaling on pipes and other surfaces.

This type of interaction could account for the enhanced ionic copper activity as a function of FA that we observed at pH 6.50. The difference in the magnitude of activities between the two experiments at this pH is probably a function of the kinetics of the system.

Based on the low concentrations of free ionic copper at pH=7.50, observed malformations rates were higher than expected. These malformations were subtle in comparison to malformations observed at lower pHs, and it is unknown if these slight anomalies would compromise survival outside the test system. Toxicity may be the result of the dissociation of other aquatic copper species such as copper hydroxides and carbonates at the low pH environment created at the gill boundary layer. Acidification of the gill boundary layer in waters of high pH occurs as a result of CO_2 excretion in fish. (Randall and Wright, 1989). Presumably, the same phenomenon occurs at the gill of aquatic amphibian larvae. Here precipitated copper may act as a source of aqueous copper as the equilibrium is shifted by copper uptake at the gills. Other copper species in solution such as hydroxides and carbonates may also be toxic to Xenopus, though probably not as toxic as the free cupric ion.

Weibull regression models fit activity-response data extremely well. One problem that we faced in fitting these models, was that we had difficulty using control data because there was no activity associated with the zero

copper groups. It was for this reason that regression models for malformation data were bound to 10% as the lower asymptote. The low millivolt data and corresponding activity data for pH 7.50 was also troublesome. The observed scatter in malformation data (Figure 6) is not surprising. Malformations were graded quantally, an individual was either malformed or not. Because there were many types of developmental anomolies observed, malformation is not a single endpoint, but a collection of endpoints. As Xenopus develop at a very rapid rate, they are very susceptable to perturbation by chemical insult.

CONCLUSION

The description of copper toxicity in terms of total copper is incomplete and simplistic. Cupric ion activity is the result of many factors that play significant roles in all aquatic systems. By measuring ionic activity, it is possible to "remove" the variables of pH and DOM, as their resulting effects are accounted for in activity measurements. As was shown in these experiments, a given concentration of total copper can be lethal or irrelevant to the development of amphibian eggs and larvae. It was also determined that FA can either increase or decrease copper activity. Although FA can complex copper and attenuate toxicity, and probably does so under most natural conditions, there are conditions under which FAs enhance toxicity. By acting as nucleation inhibitors, FAs may create stable supersaturations of ionic and other aqueous copper species, resulting in a more toxic aquatic matrix. There may also be other mechanisms for enhanced toxicity in the presence of FA.

BIBLIOGRAPHY

Allard, B., H. Boren, and A. Grimvall (eds.), 1991. Humic Substances in Aquatic and Terrestrial Environments. Springer-Verlag Publishers, Berlin. 514 pp.

Allison, J.D., D.S. Brown, and J. Novo-Gradac. 1990. MINTEQA2/PRODEFA2, a geochemical assessment model for environmental systems: Version 3.0 user's manual. Center for Exposure Assessment Modeling. Environmental Research Laboratory, U.S. Environmental Protection Agency, Athens, Georgia, USA.

American Society for Testing and Materials. Designation: E 1439 - 91. Standard Guide for Conducting the Frog Embryo Teratogenesis Assay - Xenopus.

Birge, W.J., J.A. Black, A.G. Westerman, and J.E. Hudson., 1979. The effects of mercury on reproduction of fish and amphibians. In: The biogoechemistry of mercury in the environment. Elsevier/North-Holland Biomedical Press. Nriagu ed. 629-655.

Buffle, J., F.L. Greter, and W. Haerdi., 1977. Measurement of complexation properties of humic and fulvic acids in natural waters with lead and copper ion-selective electrodes. Analytical Chemistry. 49, 216-222.

Christman, R.F., and E.T. Gjessing (eds.), 1983. Aquatic and Terrestrial Humic Materials. Ann Arbor Science, MI. U.S.A. 538 pp.

Dzombak, D.A., W. Fish, and F.M.M. Morel., 1986. Metal-humate interactions. 1. Discrete ligand and continuous distribution models. Environ. Sci. Technol., vol. 20, no. 7, 669-675.

Garvey, J.E., H.A. Owen, and R.W. Winner., 1991. Toxicity of copper to the green alga, Chlamydomonas reinhardtii (Chlorophyceae), as affected by humic substances of terrestrial and freshwater origin. Aquat. Toxicol. 19, 89-96.

Hering, J.G., and F.M.M. Morel., 1988. Kinetics of trace metal complexation: Role of Alkaline-Earth matals. Environ. Sci. Tech. vol 22, no. 12, 1469-1478.

Kramer, C.J.M. and J.C. Duinker (eds.), 1984. Complexation of Trace Metals in Natural Waters. Martinus Nijhoff/Dr. W. Junk Publishers, The Hague. 448 pp.

McKnight, D.M., G.L. Feder, E.M. Thurman, R.L. Wershaw, J.C. Westall., 1983. Complexation of copper by aquatic humic substances from different environments. The Science of the Total Environment. 28, 65-76.

Meador, J.P., 1991. The interaction of pH, dissolved organic carbon and total copper in the determination of ionic copper and toxicity. Aquat. Toxicol. 19, 13-32.

Meador, J.P., F.B. Taub, and T.H. Sibley., 1993. Copper dynamics and the mechanism of ecosystem level recovery in a standardized aquatic microcosm. Ecological Applications. 3(1), 139-155.

Nancollas, G.H. and S.J. Zawacki., 1984. in Industrial Crystallization 84, 51 eds S.J. Jacic and E.J. de Jong, Elsevier, Amsterdam.

Neter, J., W. Wasserman, and M.H. Kutner., 1983. Applied Linear Regression Models. Richard D. Irwin, Inc. Homewood Illinois. 547 pp.

Orion. 1968. Instruction manual. Cupric ion electrode, model 94-29. Orion Research, Boston, Massacuusetts, USA.

Randall, D.J., and P.A. Wright., 1988. The interaction between carbon dioxide and ammonia excretion and water pH in fish. Can. J. Zool. 67, 2936-2942.

Schnitzer, M. and H. Kerndorff., 1981. Reactions of fulvic acid with metal ions. Water, Air, and Soil Pollution. 15, 97-108.

Shanmukhappa, H. and K. Neelakantan., 1990. Influence of humic acid on the toxicity of copper, cadmium and lead to the unicellular alga Synechosystis aquatilis. Bull. Environ. Contam. Toxicol. 44, 840-843.

Spry, D.J. and J.G. Wiener., 1991. Metal bioavailability and toxicity to fish in low alkalinity lakes: A critical review. Environmental Pollution. 71, 243-304.

Stackhouse, A.R. and W.H. Benson., 1988. The influence of humic acid on the toxicity and bioavailability of selected trace metals. Aquat. Toxicol. 13, 99-108.

Stumm, W. and J.J. Morgan., 1981. Aquatic Chemistry: an introduction emphasizing chemical equilibria in natural waters. J. Wiley & Sons, New York, 780 pp.

Thurman, E.M., 1985. Organic Geochemistry of Natural Waters. Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, 497 pp.

Tipping, E., 1993. Modelling the competition between alkaline earth cations and trace metal species for binding by humic substances. Environ. Sci. Technol. 27, 520-529.

U.S. Environmental Protection Agency, 1980. Ambient water quality criteria for copper. EPA 440/5-80-036. U.S. Govt. Print Office, Washington, DC, U.S.A.

U.S. Geological Survey, 1989. Humic substances in the Suwannee River Georgia: Interactions, properties, and proposed structures. Oper file report 87-557. Denver, CO. U.S.A.

van Rosmalen, G.M., M.P.C. Weijnen, and J.A.M. Meijer., 1983. in Industrial Water Treatment and Conditioning; 36th Inter. Conf. CEBEDEAU, p. 297, Liege.

Walton, A.G., 1967. The Formation and Properties of Precipitates. Interscience Publishers. John Wiley and Sons, New York. 232 pp.

Winner, R.W., 1984. The toxicity and bioaccumulation of cadmium and copper as affected by humic acid. Aquat. Toxicol. 5, 267-274.

Winner, R.W., 1985. Bioaccumulation and toxicity of copper as affected by interactions between humic acid and water hardness. Water Res. 19, 449-455.

Winner, R.W., 1986. Interactive effects of water hardness and humic acid on the chronic toxicity of cadmuim to Daphnia pulex. Aquat. Toxicol. 8, 281-293.

Winner, R.W. and M.P. Farrell., 1976. Accute and chronic toxicity of copper to four species of Daphnia. J. Fish. Res. Board Can. 33, 1685-1691.

Winner, R.W. and J.D. Gauss., 1986. Relationship between chronic toxicity and bioaccumulation of copper, cadmium and zinc as affected by water hardness and Humic acid. Aquat. Toxicol. 8, 149-161.

APPENDICES

Appendix 1.

DISSOLVED ORGANIC MATERIAL, HUMIC AND FULVIC ACIDS

Most surface waters contain small amounts of dissolved organic material (DOM), usually ranging from 0.1 to 10 mg/l (Stumm and Morgan, 1981). In general, lakes, streams and rivers have approximately 10 times more inorganic material than organic material, while wetland systems are usually richer in organics than inorganics. This richness of organic acids gives wetland waters their characteristically low pHs (3-6). Humic materials, namely humic and fulvic acids, make up 30-80% of the DOM in most surface waters, and can make up 50-90% of the dissolved organic carbon in colored waters (Thurman, 1985 see Table 1). Humic materials are the breakdown products of plants and other biological materials. They tend to contain numerous functional groups, mainly carboxylic acids, but they also contain hydroxyls, phenolic hydroxyls, carbonyls, and carbohydrates. These functional groups are thought to be the sites of metal complexation in natural waters (see Appendix 1 for hypothetical structures of fulvic acid).

Fulvic acid is defined as the water soluble portion of humic material that remains in solution under acidic conditions. Fulvics are generally smaller than humic acids,

with molecular weights ranging from 500-2000 Daltons. In most surface waters fulvic acids are more highly represented than the larger true humic acids. In some wetland waters, fulvic acid can make up 90% of the dissolved organic material. The soluble nature of fulvic acids is probably due to the higher percentage of carboxylic acids on the molecule and its smaller size.

WATER TYPE	DOC of HUMICS (mg/l)
Ground water	0.05 - 0.10
Seawater	0.10 - 0.25
Stream	0.5 - 2.0
Lake	1.0 - 4.0
River	1.0 - 4.0
Wetlands	10 - 30

Table 4. Relative abundance of humic materials in surface waters (Thurman, 1985).

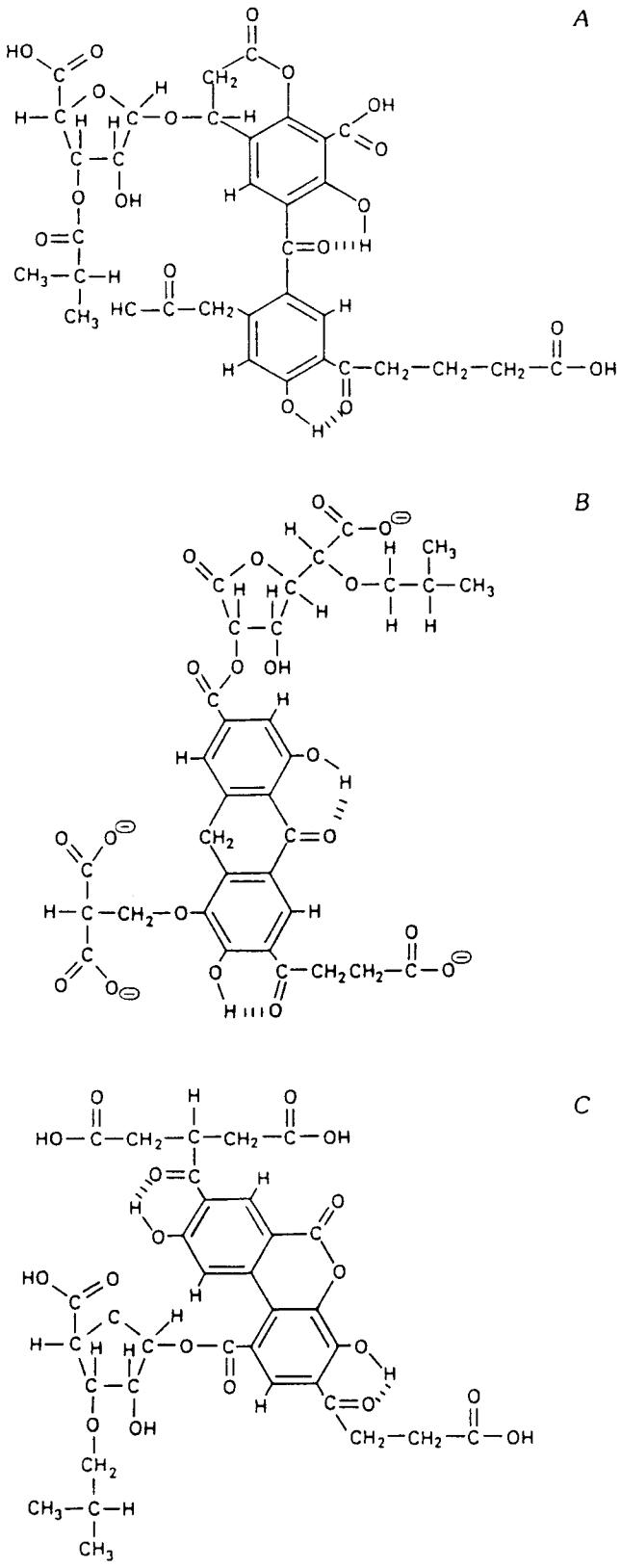


Figure 7.--Three proposed average structural models (A, B, and C) of the fulvic acid.

Appendix 2.

BIOASSESSMENT

The Frog Embryo Teratogenicity Assay - Xenopus (FETAX) is a standardized toxicity test (ASTM 1993) which evaluates developmental toxicity of single compounds or complex mixtures on whole embryos and early larval stages of the South African Clawed Frog - Xenopus laevis. The assay utilizes various aspects of this species' biology to yield meaningful results in 96-hour duration tests. Xenopus can be hormonally induced to breed throughout the year, which is extremely useful in a laboratory situation, especially since North American species are seasonal breeders and are often difficult to rear in captivity. The declining natural stocks of native species throughout the country also make field collection of eggs difficult and undesirable. Xenopus develop at a very rapid rate, and at 96 hours are transparent free swimming tadpoles. Examination of developmental abnormalities is easily done with a dissecting scope, making FETAX a good test system for studying sublethal effects of various contaminants. As amphibians appear to be declining on a global scale, the FETAX assay is a valuable tool for examining the effects of heavy metals on amphibians in different aquatic environments.

Appendix 3.

DILUTION WATER

	mg/l	MOL. WT.	MOLARITY
$CaCl_2 * 2H_2O$	131.605	147.019	6.76 E-4
$MgSO_4$	36.63	120.374	3.04 E-4
$NaHCO_3$	100.00	84.007	1.19 E-3
KH_2PO_4	136.10	136.089	1.00 E-3

Chemical composition

Ca	27.1
Mg	8.0
Na	30.7
K	44.0
Cl	52.3
HCO_3	72.6
SO_4	29.2
PO_4	94.9

Appendix 4.

		DAY1	DAY2	DAY3	DAY4
pH	5.50				
	MEAN	5.84	5.49	5.30	5.23
	SE	.06	.13	.15	.27
	MIN	5.72	5.27	5.10	5.00
	MAX	5.93	5.76	5.77	5.87
pH	7.50				
	MEAN	7.47	7.37	7.30	
	SE	.02	.02	.01	
	MIN	7.41	7.31	7.28	
	MAX	7.51	7.42	7.33	
pH	6.50				
	MEAN	6.75	6.59	6.52	6.64
	SE	.05	.06	.05	.03
	MIN	6.68	6.46	6.41	6.57
	MAX	6.85	6.69	6.61	6.70

Table 5. pH drift. Pooled pH data for all dishes following a 24 hour exposure period. The initial pHs were within .04 units from the desired starting pH each day.

Appendix 5.

pH	FA (mg/l)	TCU (mg/l)	mV	ICU (mg/l)	LOG Cu^{+2} ACTIVITY
5.50	0.00	.049	97.5	.044	-6.2865
		.075	105.8	.068	-6.1016
		.156	115.7	.141	-5.7836
		.414	128.2	.375	-5.3598
		1.108	141.6	1.004	-4.9326
	0.50	.044	94.3	.034	-6.4496
		.085	106.1	.069	-6.0648
		.156	113.3	.116	-5.8301
		.414	127.2	.346	-5.3769
		1.095	141.9	1.028	-4.8976
	5.00	.047	88.7	.022	-6.6322
		.090	100.1	.043	-6.2605
		.143	108.5	.079	-5.9866
		.422	122.2	.232	-5.5399
		1.096	138.1	.758	-5.0215
6.50	0.00	.044	92.4	.023	-6.5567
		.071	98.1	.037	-6.3489
		.162	111.6	.085	-5.9908
		.434	114.9	.172	-5.6869
		1.190	113.8	.173	-5.6835
	0.50	.033	88.8	.017	-6.6289
		.071	96.3	.032	-6.3827
		.162	109.2	.069	-5.9638
		.437	120.8	.288	-5.5855
		1.199	119.9	.296	-5.6149
	5.00	.033	86.3	.013	-6.7104
		.073	87.1	.014	-6.6843
		.164	100.5	.032	-6.2474
		.433	118.0	.226	-5.6752
		1.193	124.8	.458	-5.4551

Table 6. Interactions. The influence of pH and fulvic acid (FA) on millivolt measurements, and estimates of free ionic copper (ICU), and cupric ion activities. For zero fulvic acid groups, ionic copper was determined from MINTEQ modelling output. The differences between voltages in the zero FA groups and the FA groups were used as "correction factors" in the equation:

$$Cu^{2+} = Cu^{2+seed}/e^{((mVseed-mVobs)/slope)*2.3}$$

to estimate free ionic copper in the fulvic acid treatment groups. A calibration curve, utilizing model output for cupric ion activities for zero fulvic acid groups was used to convert all other voltage measurements into activities.